The Patent Office

INVESTOR IN PEOPLE

PRIORITY DOCUMENT
SUBMITTED OR TRANSMITTED IN
COMPLIANCE WITH
RULE 17.1(a) OR (b)

The Patent Office
Concept House
Cardiff Road
Newport
South Wales
NP10 8QQ

I, the undersigned, being an officer duly authorised in accordance with Section 74(1) and (4) of the Deregulation & Contracting Out Act 1994, to sign and issue certificates on behalf of the Comptroller-General, hereby certify that annexed hereto is a true copy of the documents as originally filed in connection with the patent application identified therein.

In accordance with the Patents (Companies Re-registration) Rules 1982, if a company named in this certificate and any accompanying documents has re-registered under the Companies Act 1980 with the same name as that with which it was registered immediately before re-registration save for the substitution as, or inclusion as, the last part of the name of the words "public limited company" or their equivalents in Welsh, references to the name of the company in this certificate and any accompanying documents shall be treated as references to the name with which it is so re-registered.

In accordance with the rules, the words "public limited company" may be replaced by p.l.c., plc, P.L.C. or PLC.

Re-registration under the Companies Act does not constitute a new legal entity but merely subjects the company to certain additional company law rules.

Signed Stephen Hordley

Dated 4 August 2003

Patents Form 1/77

Act 1977
16)

THE PATENT OFFICE
10 JUL 2002
RECEIVED BY HAND

The Patent Office

1/77

11JUL02 E748472-4 002870
P01/7700 0.00-0216026.5

Request for grant of a patent

(See the notes on the back of this form. You can also get an explanatory leaflet from the Patent Office to help you fill in this form)

The Patent Office

Cardiff Road
Newport
South Wales
NP9 1RH

1. Your reference — REP07135GB

2. Patent application number *(The Patent Office will fill in this part)* — 0216026.5

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

Cambridge Biotechnology Ltd.
10 Wellington Street
Cambridge
CB1 1HW

Patents ADP number *(if you know it)* — 8208761001

If the applicant is a corporate body, give the country/state of its incorporation — United Kingdom

4. Title of the invention — NUCLEIC ACID AMPLIFICATION METHOD

5. Name of your agent *(if you have one)* — Gill Jennings & Every

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*

Broadgate House
7 Eldon Street
London
EC2M 7LH

Patents ADP number *(if you know it)* — 745002

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application number *(if you know it)*	Date of filing *(day / month / year)*

7. If this application is divided or otherwise derived from an earlier UK application, give the number and the filing date of the earlier application

Number of earlier application	Date of filing *(day / month / year)*

8. Is a statement of inventorship and of right to grant of a patent required in support of this request? *(Answer 'Yes' if:*

a) any applicant named in part 3 is not an inventor, or
b) there is an inventor who is not named as an applicant, or
c) any named applicant is a corporate body.
See note (d))

YES

Patents Form 1/77

Enter the number of sheets for any of the following items you are filing with this form. Do not count copies of the same document

Continuation sheets of this form	
Description	6
Claim(s)	1
Abstract	
Drawing(s)	1

10. If you are also filing any of the following, state how many against each item.

Priority documents	
Translations of priority documents	
Statement of inventorship and right to grant of a patent *(Patents Form 7/77)*	
Request for preliminary examination and search *(Patents Form 9/77)*	
Request for substantive examination *(Patents Form 10/77)*	
Any other documents *(please specify)*	NO

11. For the applicant
Gill Jennings & Every

I/We request the grant of a patent on the basis of this application.

Signature

Date
10 July 2002

12. Name and daytime telephone number of person to contact in the United Kingdom

R E Perry
020 7377 1377

Warning

After an application for a patent has been filed, the Comptroller of the Patent Office will consider whether publication or communication of the invention should be prohibited or restricted under Section 22 of the Patents Act 1977. You will be informed if it is necessary to prohibit or restrict your invention in this way. Furthermore, if you live in the United Kingdom, Section 23 of the Patents Act 1977 stops you from applying for a patent abroad without first getting written permission from the Patent Office unless an application has been filed at least 6 weeks beforehand in the United Kingdom for a patent for the same invention and either no direction prohibiting publication or communication has been given, or any such direction has been revoked.

Notes

a) If you need help to fill in this form or you have any questions, please contact the Patent Office on 0645 500505.

b) Write your answers in capital letters using black ink or you may type them.

c) If there is not enough space for all the relevant details on any part of this form, please continue on a separate sheet of paper and write "see continuation sheet" in the relevant part(s). Any continuation sheet should be attached to this form.

d) If you have answered 'Yes' Patents Form 7/77 will need to be filed.

e) Once you have filled in the form you must remember to sign and date it.

f) For details of the fee and ways to pay please contact the Patent Office.

NUCLEIC ACID AMPLIFICATION METHOD

Field of the Invention

This invention relates to a nucleic acid amplification method.

Background of the Invention

WO01/06004 discloses a method for increasing the number of polynucleotides containing sequences corresponding to a mRNA species present in a sample. This method comprises 3 steps, i.e. reverse transcription of the mRNA species using a first heeled primer population, to provide first cDNA strands; synthesis of second cDNA strands from the first, using a second heeled primer population; and amplification of the first and second cDNA strands. The heel sequence of the primers used in the first and/or second steps contains a RNA polymerase promoter site. The primers used in the amplification comprise at least a portion of the first heel sequence and a portion of the second heel sequence. Either or each of the heel sequences preferably includes the nucleotide sequence of a restriction enzyme cleavage site; the polynucleotides that are produced may include concatomers, but can be treated with an agent that cleaves at this cleavage site, without affecting the desired amplicons.

Summary of the Invention

The present invention is based on the discovery that the procedure disclosed in WO01/06004 can be simplified. According to the present invention, a method for increasing the number of polynucleotides containing sequences corresponding to a mRNA species present in a sample, comprises the steps of:

(i) reverse transcription of the mRNA species using a heeled 5'-amplification primer (FAP) and a heeled 3'-amplification primer (TAP), wherein each primer sequence is unique and absent from the relevant genome, and each heel sequence includes a RNA polymerase promoter site, and the FAP includes a variable sequence, whereby the RNA is reverse-transcribed to produce double-stranded cDNA and then multiple cDNAs according to the variable sequence; and

(ii) amplification of the cDNA using primers sufficiently complementary to FAP and TAP.

It will be evident that, in the present invention, reverse transcription and production of multiple cDNAs are conducted in one step. While the present invention

shares with the known procedure the use of a randomer sequence, so that the use of appropriate populations of varied FAP/TAP sequences gives multiple cDNAs, the novel procedure is simpler. The reverse transcription proceeds more efficiently, and fewer PCR cycles are required.

Further, by comparison with the known procedure, the need for rare restriction sites is avoided. The production of complex products is minimised, due in part to the use of unique sequences in FAP and TAP which are absent from the genome being investigated.

The present invention readily allows the inclusion of specific restriction sites, for manufacturing subtracted normalised and enriched cDNA libraries. It also allows the inclusion of specific restriction sites for lambda cloning, for the manufacture of single cell libraries. Again, specific restriction sites can be included, for fragmentation, for use as probes on the microarrays and filters. The procedure can readily be linked to a protocol for laser capture microdissection, e.g. as described by Fend *et al*, Am J Pathol. (1999)154(1):61-6.

While bearing in mind the key distinctions between the present invention and the method disclosed in WO01/06004, the disclosure of the latter document is largely relevant, and is incorporated herein by reference.

An embodiment of the invention comprises a third step, i.e. (iii) transcription, to produce RNA run-offs from either end of the amplicons, e.g. using T3 RNA polymerase or T7 RNA polymerase. This is particularly suitable for use *in vitro* and when the number of cells is small.

Description of Preferred Embodiments

The following description is of a procedure (abbreviated as "MEX") that illustrates the invention. As above, "FAP" and "TAP" are used as abbreviations for 5'- and 3'-amplification primers, respectively.

1) MEX RT reaction.

In this step, specific priming sites for PCR amplification were engineered into appropriate ends of the cDNA population.

10µl of total RNA (prepared either by standard molecular biology protocols from cells/tissues/Laser Capture Microscopy cell harvests or from direct patch clamp harvesting) dissolved in water was added to 1µl of each of FAP-Randomer, i.e. ACT CCG

GGA ACC ATA GTG GCA CTC CTA ATT AAC CCT CAC TAA AGG GAG ATC GTAC $(N)_{15}$, and TAP-RT, i.e. CAA GCA TTC AGT ATC TCG ACT GTA ATA CGA CTC ACT ATA GGG AGA GAT CGT AC $(T)_{20}$, each at 10μM. This was heated to 70°C for 5 minutes before snap-cooling on ice for 2 minutes.

4μl of 5x Reverse Transcription buffer (Superscript II kit, Invitrogen), 2μl of 0.1M dithiothreitol (DTT, Superscript II kit, Invitrogen), 1μl of dNTPs (10mM mixture of each of dATP, dTTP, dGTP and dCTP), and 1μl of Superscript II reverse transcriptase enzyme (Superscript II at 200U/μl, Invitrogen) were added, and the reaction incubated at 42°C for 1 hour.

2) MEX amplification reaction

In this step, the reverse transcribed MEX-RT products were amplified for downstream molecular applications by PCR.

5ul of the RT reaction was amplified in a 100 μl PCR by the addition of the following reagents: 10μl Advantage II PCR buffer (Clontech), 1μl of dNTPs (10mM mixture of each of dATP, dTTP, dGTP and dCTP), 1μl FAP, i.e. ACT CCG GGA ACC ATA GTG GCA CTC C, at 10μM and 1ul TAP, i.e. CAA GCA TTC AGT ATC TCG ACT G, at 10μM and 77μl of sterile water.

Cycling parameters were as follows: 95°C for 1 minute, for one cycle, then up to 24 cycles of 95°C for 15 seconds, 65°C for 30 seconds, 68°C for 6 minutes.

MEX Mechanism of action

In the first step, a 3'-heeled primer (TAP) initiates reverse transcription and first round cDNA synthesis through binding of a $(T)_{20}$ sequence, contained in the 3'-terminus of the primer, to polyA tails in the mRNA population. This primer contains a heel sequence for subsequent PCR amplification and a nested RNA polymerase promoter site (for T7 RNA polymerase) for subsequent *in vitro* transcription reactions. A second primer (FAP) is also included in the RT reaction; this initiates second round cDNA synthesis through semi-randomly priming from the first strand cDNA via a $(N)_{15}$ sequence incorporated at the 3'-terminus of the primer. This primer also contains a heel sequence for subsequent PCR amplification (NB This heel sequence is distinct from that in the 3'-primer) and a nested RNA polymerase promoter site (for T3 RNA polymerase) for

subsequent *in vitro* transcription reactions. The heel sequences are unique sequences as defined by their absence from the current genome databases.

In this way, a population of mRNAs is converted to double-stranded cDNAs that are anchored at their 3'-termini to the TAP and at their 5'-termini to the FAP.

The cDNA population therefore contains the following species of MEX fragments (where Gene X is any gene that has been reverse transcribed):

FAP SEQUENCE - T3 RNA POLYMERASE SEQUENCE $-(N)_{15}$ - GENE X $-(T)_{20}$-T7 RNA POLYMERASE - TAP SEQUENCE

MEX from control RNAs

Approx. 100ng of total brain RNA was reverse transcribed according to the MEX protocol described above. This was serially diluted and subjected to MEX amplification for either 9 cycles or 15 cycles. In addition, a control, where no amplification was performed, was also included. PCR was performed to determine the degree of amplification of actin mRNA transcripts in the cDNA and MEX amplified pools.

Actin transcripts could only be detected in unamplified cDNAs at a concentration of 50ng of total RNA equivalent. Transcripts could not be detected at cDNA concentrations below 5pg. In contrast, actin could be detected in the same cDNA samples at concentrations as low as 0.5pg after 9 cycles of MEX amplification and 50fg after 15 cycles of MEX amplification.

This experiment demonstrates that MEX amplification of cDNAs extends the range of detection beyond that where detection is possible by conventional RT PCR alone.

MEX from Laser Capture Cells

G-1 cultured cells were grown on glass microscope slides and processed for Laser Capture Microdissection (LCM). Defined numbers of cells were harvested, and RNA extracted using the Stratagene mico-RNA extraction kit. This RNA was reverse-transcribed using MEX RT primers and then subjected to MEX amplification for either 15 or 21 cycles. A proportion of the cDNA was also retained for analysis without MEX amplification. PCR was performed, using primers specific for actin, as the final assay of the efficiency of the MEX amplification.

Actin sequence was detected in as few as 50 cells after 15 cycles of MEX amplification and in as few as 10 cells after 21 cycles of MEX amplification. In contrast,

actin sequence was undetectable in samples that were not subject to MEX pre-amplification.

In conclusion, MEX can be used to link molecular techniques to LCM.

MEX linked to *in vitro* transcription

Approx. 100ng of total RNA was reverse-transcribed using MEX primers, serially diluted and subsequently MEX amplified for three, six or nine cycles. These products were *in vitro* transcribed (IVT) using the Megascribe kit (Ambion) and reverse-transcribed for a second time using a conventional $(dT)_{18}$ primer and Superscripts II (Invitrogen). To gauge the relative efficiency of the amplifications, 30 cycles of gene specific PCR for the actin sequence were performed on these products. The combination of three cycles of MEX, when used in conjunction with IVT, was sufficient to enable the identification of actin transcripts in 5pg of total RNA. The application of an additional three cycles of MEX prior to IVT enabled this detection window to be extended to 50fg of total RNA. For comparison, 15 cycles of MEX were required to enable detection of actin in 50fg of RNA without the use of IVT.

In conclusion, MEX can be used in consort with IVT to extend the sensitivity of the detection, whilst minimizing cycle number. This may be important for increasing the amplicon yield from MEX whilst retaining linearity of representation in the amplicon pool. In addition, the production of RNA run-offs from MEX products allows the use of these products as probes on Affymetrix and other microarrays.

MEX used in Target Identification

To demonstrate the utility of MEX, c-fibres were harvested using LCM from an *in vitro* model of pain. This model was based on primary cultures of rat trigeminal ganglion cells treated with pro-nociceptive agents such as Nerve Growth Factor (NGF). RNA from these harvests was reverse-transcribed and amplified using 24 cycles of MEX. The resulting amplicon pool was then assayed with primers for genes with known association with nociception. When the amplicon pool was assayed prior to, and following, MEX amplification a distinct difference could be observed. Whilst control genes such as actin and cyclophilin (act and cyc) could be detected in the pre-MEX pool, other less abundant and biologically more important genes for the nociceptive response, i.e. TrkB receptor, PN3, sodium channel Beta 3 subunit, calcitonin gene related protein, vasointestinal peptide, neuropeptide Y, galanin and tyrosine hydroxylase (PN3, β3, CGRP,

VIP, NPY, Gal, and TH) were absent. This was in contrast to the amplicon pool following MEX amplification, where the majority of these pro-nociceptive genes were readily detectable. This also validated the *in vitro* model of pain.

Therefore, MEX is of use in detecting genes of interest in small numbers of pathologically relevant cells. These genes may be used as targets for therapeutic intervention.

MEX used in library construction

The MEX primers have been designed to be compatible with many downstream applications. These include use in the manufacture of subtracted PCR libraries.

MEX products from small numbers of cells (100ng total RNA equivalent) were amplified from trigeminal ganglion primary cultures treated with or without nerve growth factor and purified to produce the starting material for the manufacture of subtracted libraries (top left hand panel). These were size-selected to remove small products and primer sequences (<50 base pairs). Linker molecules were ligated to these products to facilitate the subtraction (these were quality controlled in the middle top panel by using combinations of linker based / gene base PCR primers) and two rounds of suppression PCR subtraction applied to the MEX amplicons. This produced the final forward and reverse subtracted library pairs (shown in the top right hand panel). The libraries were quality controlled to ensure that "housekeeper" genes common to both subtraction pools had been removed during the subtraction process. This was demonstrated by the removal of actin transcripts from the subtracted libraries (bottom left hand panel). As a final check of the content of these libraries, products were cloned and sized (bottom right hand panel). A number of these clones were sequenced and differential expression was identified.

Therefore, MEX can be used to analyse gene expression in small numbers of cells and to identify genes that are differentially expressed between two or more cell types or cell types with distinct phenotypes.

CLAIMS

1. A method for increasing the number of polynucleotides containing sequences corresponding to a mRNA species present in a sample, the method comprising the steps of:

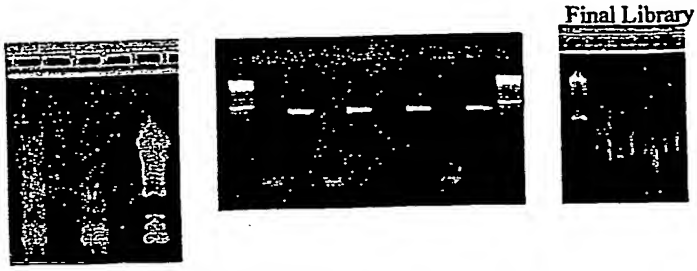
(i) reverse transcription of the mRNA species using a heeled 5'-amplification primer (FAP) and a heeled 3'-amplification primer (TAP), wherein each primer sequence is unique, and either or each heel sequence includes a RNA polymerase promoter site, and the FAP includes a variable sequence, whereby the RNA is reverse-transcribed to produce double-stranded cDNA and then multiple cDNAs according to the variable sequence; and

(ii) amplification of the cDNA using primers sufficiently complementary to FAP and TAP.

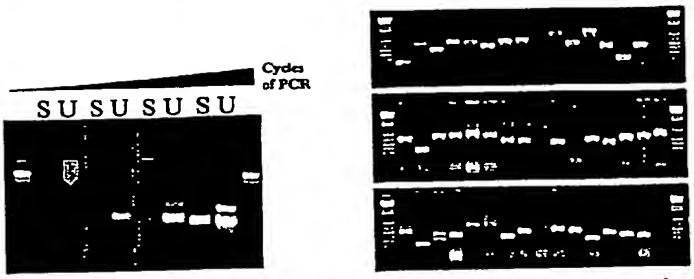
2. A method according to claim 1, which additionally comprises the step of:

(iii) transcription, to produce RNA run-offs from either end of the amplicons.

MEX Used in library construction



- MEX has been used to construct a PCR subtracted library, top panels, left raw amplicons, middle pre subtraction products and right final library).
- This library is enriched for key marker genes (bottom left panel) and contains clones that are known to be differentially expressed a s a consequence of the treatment



MEX has been used to make a PCR subtracted library

INTERNATIONAL SEARCH REPORT

Internat' Application No
PCT/GB 03/02989

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 06004 A (UNIV CAMBRIDGE TECH; COX PETER (GB); RICHARDSON PETER (GB)) 25 January 2001 (2001-01-25) cited in the application the whole document	1-15,17, 18
A	WO 00 08208 A (MEDICAL RES COUNCIL; RICHARDSON PETER JOHN (GB); DIXON ALISTAIR K) 17 February 2000 (2000-02-17)	
P,X	EP 1 275 738 A (HOFFMANN LA ROCHE ;ROCHE DIAGNOSTICS GMBH (DE)) 15 January 2003 (2003-01-15) the whole document, in particular figure 1	1,2,6, 14,15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 October 2003	13/10/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pinta, V

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Information on patent family members

Internat'l Application No
PCT/GB 03/02989

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 0106004	A	25-01-2001	AU	6695600 A	05-02-2001
			CA	2378070 A1	25-01-2001
			WO	0106004 A2	25-01-2001
			EP	1196639 A2	17-04-2002
			JP	2003505034 T	12-02-2003
WO 0008208	A	17-02-2000	AU	5294399 A	28-02-2000
			WO	0008208 A2	17-02-2000
EP 1275738	A	15-01-2003	EP	1275734 A1	15-01-2003
			EP	1275738 A1	15-01-2003
			JP	2003093058 A	02-04-2003
			US	2003113754 A1	19-06-2003

INTERNATIONAL SEARCH REPORT

Internat' Application No
PCT/GB 03/02989

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12Q1/68 C12N15/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C12Q C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, PAJ, EMBASE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 01 06004 A (UNIV CAMBRIDGE TECH; COX PETER (GB); RICHARDSON PETER (GB)) 25 January 2001 (2001-01-25) cited in the application the whole document	1-15,17, 18
A	WO 00 08208 A (MEDICAL RES COUNCIL; RICHARDSON PETER JOHN (GB); DIXON ALISTAIR K) 17 February 2000 (2000-02-17)	
P,X	EP 1 275 738 A (HOFFMANN LA ROCHE ;ROCHE DIAGNOSTICS GMBH (DE)) 15 January 2003 (2003-01-15) the whole document, in particular figure 1	1,2,6, 14,15

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
7 October 2003	13/10/2003

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Pinta, V

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

tion on patent family members

Internat. Application No
PCT/GB 03/02989

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 0106004 A	25-01-2001	AU 6695600 A	05-02-2001
		CA 2378070 A1	25-01-2001
		WO 0106004 A2	25-01-2001
		EP 1196639 A2	17-04-2002
		JP 2003505034 T	12-02-2003
WO 0008208 A	17-02-2000	AU 5294399 A	28-02-2000
		WO 0008208 A2	17-02-2000
EP 1275738 A	15-01-2003	EP 1275734 A1	15-01-2003
		EP 1275738 A1	15-01-2003
		JP 2003093058 A	02-04-2003
		US 2003113754 A1	19-06-2003